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Received September 5, 1961 P.S.E.B.M., 1961, v108

Action of Ammonium Ion on an Early Phase of Viral Infection.* (27060)

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In tumor cells influenza virus produces high titers of hemagglutinins but little infectious virus(1). This indicates an incomplete replication cycle with no spread of virus among cells. A previous report has shown that Krebs 2 ascites cells liberate ammonia from glutamine, and the antiviral effect of immediate or delayed treatment with these substances was described(2). The purpose of the investigations reported here was to obtain information about the nature of the initial process of infection during which there is a short period when viral growth may be inhibited by addition of ammonium ions to the medium. Later treatment is without effect.

Method. The medium, the method of preparing the ascites cell suspensions, and determination of cell-associated hemagglutinins have been described(2). To suspensions containing 3.2×10^6 tumor cells per ml the PR_8 strain of virus was added at a concentration of 16 to 32 hemagglutinating units (HA) per ml, giving an input multiplicity of the order of 10 E.I.D.50 per cell. Cells were allowed to adsorb virus for definite periods of time, then washed and changed to fresh medium. If a single cycle of viral replication is assumed, the HA yield reflects the proportion of cells originally infected. As judged by HA titers at 24 hours about 15% of susceptible cells were infected within 10 minutes, and virtually 100% between 2 and 4 hours. Reducing the temperature of incubation from 35°C to 4°C caused only a slight diminution in rate of viral attachment to cells. Maximum HA titers obtainable in this system were 1,000 to 2,000 units per 0.1 g of extracted cells.

Ammonium phosphate was added at a concentration of 0.01% and unless otherwise noted remained in the medium during the subsequent incubation period. Disappearance of NH_3 nitrogen from the fluid indicated an uptake by cells during the first 4 hours of about 100 μg per gram wet weight.

Results. To determine the length of the period of sensitivity to NH_4^+, cells and virus were kept at 4°C for 2 hours, then the cells were washed and suspended in medium warmed to 35°C. In the tubes receiving ammonium phosphate at 10 minutes the 24-hour HA titers were 32, in those treated at 15 minutes 128, and at 40 minutes 256 (control titer 512). This experiment shows quite clearly that at 35°C the period after adsorption during which viral growth may be significantly inhibited by NH_4^+ is only a few minutes.

When cells are kept with virus at 5°C or 15°C for a period as long as 4 hours then washed and treated immediately with NH_4^+, marked inhibition of HA formation is obtained (Table I). This indicates that low temperature prolongs the period of NH_4^+ sensitivity in contrast to its slight effect on attachment of virus. At both 25 and 35°C, however, the period of highest NH_4^+ sensitivity is about 10 to 20 minutes.

Since low temperature delays the process by which virus becomes refractory to NH_4^+, active metabolism of the host cell is probably a necessary factor in this reaction. Omitting the substrate during preincubation did not

* These studies were aided by Research Grant from U.S.P.H.S. and by funds from Eugene Higgins Trust.

TABLE I. Period of ammonium sensitivity as affected by temperature, medium, and inhibitors.

Preincubation with virus Time hr	Temp. °C	Medium*	HA titer after 24 hr with NH_4^+ as % of control
4	5	gl + gc	0.4
4	15	" "	1.5
¼	25	" "	3
1	25	" "	100
¼	35	" "	< 0.4
¾	"	" "	100
1	"	BSS only	50
2	"	gl + CN 60	< 3
2	"	gm + DNP	< 6
1	"	gl + CN 30	3
4	"	" " "	25
1	"	gl + gc + CN 60	100
1	"	" " + DNP	100

* Hanks' balanced salt solution (BSS) with substrates and inhibitors as follows: gl, glutamate 1 mg/ml; gc, glucose 1 mg/ml (with bicarbonate at 0.14%); gm, glutamine 1 mg/ml; CN, potassium cyanide (pH 7.6) 30 or 60 μg/ml; DNP, 2-4 dinitrophenol 50 μg/ml.
$(NH_4)_2 HPO_4$ at 0.01% in all experiments.

appreciably increase the period of NH_4^+ sensitivity, probably because the cells contain a reserve of metabolites. Consequently studies were done with cyanide and 2-4 dinitrophenol in media with and without glucose to determine whether oxidative or glycolytic sources of energy were essential. Cells were incubated with virus in the presence of these inhibitors for short periods then washed and suspended in the glutamate-glucose medium. To one set of tubes containing cells which had been infected in the presence of cyanide or DNP ammonium phosphate was added. The other sets containing treated infected cells received no NH_4^+ and served as controls for the effect of the inhibitors by themselves. In these the HA yields reached 128 to 256, indicating only a slight reduction in titer from CN or DNP alone. In the cell virus system first treated in absence of glucose with cyanide at 60 μg/ml or DNP at 50μg/ml for 2 hours then placed in a medium containing NH_4^+ no hemagglutinins were formed (Table I). Cyanide at 30 μg/ml under similar conditions also increased the period of NH_4^+ sensitivity. Interference with oxidative metabolism of the cells, like low temperature, has the effect of prolonging the period of NH_4^+ sensitivity. When, however, the preincubation medium contained glucose with cyanide or DNP, the period of NH_4^+ sensitivity was less than an hour (last lines of Table I).

To test for reversibility of the effect of NH_4^+, cells, virus, and ammonium phosphate were incubated together for varying periods of time from 1 to 18 hours. The cells were then washed and suspended in fresh medium without virus or NH_4^+ for a further incubation period of 24 to 48 hours. As the results presented in Table II show, even after an initial period of a few hours' exposure to NH_4^+ there is interference with subsequent HA formation, and at 18 hours most of the virus capable of replication seems to have been lost. As shown previously, cells incubated by themselves with NH_4^+ for 24 hours retain the ability of supporting some viral growth and ammonium phosphate at a concentration of 0.01% does not reduce the infective property of extracellular virus(2).

TABLE II. Reversibility of effect of ammonium ion and action of antibody.

Period of exposure to NH_4^+. (hr)*	HA titer** a. NH_4^+.	b. Control	a/b × 100
1	128	256	50
2	256	1024	25
4	128	2048	6
18	4	1024	1.5
4 (ab 50)	<4	32	—
4 (ab 10)	32	512	—

* Virus added at 0 hr.
** 24 to 48 hr after removal from medium with NH_4^+. Ab 50, ab 10 indicate that NH_4^+-treated infected cells and controls were placed in fresh medium containing 50 or 10 HA-inhibiting units of antibody.

The poorly defined mechanism by which animal viruses reach the interior of cells may involve ingestion (pinocytosis) or enzymatic activity. To ascertain if the presence of NH_4^+ caused attached virus to remain on the surface and hence accessible to antibody, cells were incubated for 4 hours with virus and NH_4^+, then placed in medium containing 10 to 50 HA-inhibiting units of antibody.† Reduction by antibody of cell-associated HA after 24 hours' incubation appeared to be similar in degree in NH_4^+-treated cells and controls (compare lines 3, 5, and 6, Table II) indicating that NH_4^+ did not increase the proportion of neutralizable virus. This procedure has been used to demonstrate delayed pene-

† Before extraction for titration of cell-associated hemagglutinins cells were routinely washed twice(1).

tration of virus in other studies(3). Also receptor-destroying enzyme has been used by Wagner(4) to show the temperature dependence of the penetration phase.

With influenza in minced chorioallantoic membrane a similar early suppression may be demonstrated by using higher concentrations of NH_4^+; but the increased NH_4^+, even when added after 4 hours, reduces HA yields to about 25% of the control. This is attributed to a toxic effect during the subsequent 24-hour incubation period.

Discussion. Jensen, Force and Unger(5) describe with influenza virus in another species of cell results essentially similar to ours(2). Using a stable line of epithelial cells originating from dog kidney, they were able to demonstrate protection by ammonium ions against the cytopathic effect of the PR8 strain. Similarly glutamine from which ammonia is formed will in the presence of bicarbonate protect Krebs 2 cells against the oncolytic action of influenza virus(2,6). These observations suggest that NH_4^+ at appropriate concentration may have a chemoprophylactic effect in certain cell-virus systems maintained *in vitro*. The concentration of NH_4^+ of 0.005% to 0.01% found to be non-toxic to cells in tissue culture but inhibitory for virus(5) is, however, greatly in excess of tolerated blood levels in the intact animal.

The ammonia effect seems to be most easily demonstrable under conditions favoring formation of large amounts of incomplete virus, although reduction of infectivity titers has been reported(5). Preliminary results with cultures of chorioallantoic membrane and influenza virus suggest that suppression of viral growth by NH_4^+ or glutamine may also occur in cells which produce complete infectious virus.

The process affected by NH_4^+ seems to require cellular energy production, but when oxidative metabolism is inhibited the requisite energy appears to be furnished by glycolysis. Because of the short duration of the period of NH_4^+ sensitivity after adsorption of virus, consideration can be given only to a mechanism which involves initial integration of the viral inoculum with cytoplasmic or nuclear components of the host cell. Ammonia may modify a biosynthetic process essential to viral invasion.

Summary. Following adsorption of influenza virus by Krebs 2 ascites cells there is a brief period of sensitivity to inhibition of viral replication by ammonia. After an hour at 35°C addition of ammonium phosphate causes no inhibition. The period of ammonium sensitivity is prolonged by low temperature, cyanide, or 2-4 dinitrophenol, but the presence of glucose in the medium prevents this effect of CN and DNP. The results suggest that the ammonium-sensitive phase of infection is an energy-requiring process which occurs soon after adsorption and penetration, and comprises initial integration of the viral inoculum with cell components.

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Received September 11, 1961 P.S.E.B.M., 1961, v108

Transfer of Allergic Encephalomyelitis Using Splenectomized Albino Rats.** (27061)

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Allergic encephalomyelitis (AE), induced in rats and other animals by injection of spinal cord plus Freund's adjuvant, has been under study in our laboratory as a model for

** This work supported by U. S. Public Health Service Grant.

* Recipient of Investigatorship of Health Research Council of City of New York.